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Review

# Engineering the ovarian niche: Environmental control of folliculogenesis in vitro

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ABSTRACT

Advancements in cancer therapies have significantly improved patient survival, but gonadotoxic treatments often compromise fertility, particularly in female patients. While ovarian tissue cryopreservation and transplantation are well-established fertility preservation options, they are not recommended for patients with blood-borne cancers or highly metastatic malignancies due to the risk of ovarian involvement. In these cases, follicle in vitro culture offers a promising alternative. However, folliculogenesis is a complex process that requires meticulous environmental control to mimic the ovarian niche. Key factors include biochemical signals delivered through culture media, biophysical support provided by three-dimensional biomaterials or the native extracellular matrix, and crucial cellular interactions that drive follicular development. Recent advances in biomaterial design have led to the creation of scaffolds that not only preserve structural integrity but also facilitate nutrient exchange and cell communication. Moreover, dynamic culture systems have shown superior outcomes compared to static models, offering a more physiologically relevant environment. This review explores the interplay of biochemical, biophysical, and mechanical factors in in vitro folliculogenesis. By synthesizing current innovations in scaffold design, culture systems, and bioactive supplementation, we outline key strategies for optimizing in vitro follicular development. These advances pave the way toward safer and more effective fertility preservation approaches for patients at high risk of ovarian metastasis and offer broader insights into reproductive biology and regenerative medicine. However, to fully realize this potential, further standardization, long-term safety studies, and critical evaluation of emerging technologies remain essential to enable robust clinical translation and personalized reproductive applications.

## 1. Introduction

The ovaries are central to female reproductive function, serving as the site for both oocyte development and the production of key steroid hormones that regulate the menstrual cycle and fertility [1]. While advances in chemotherapy and radiotherapy have significantly improved survival rates among young female cancer patients [2], these treatments are often gonadotoxic, leading to substantial damage to ovarian follicles, the fundamental functional units of the ovary [3]. As a result, fertility preservation has become an essential consideration in the management of cancer in reproductive-aged and prepubertal females. Among the available options, ovarian tissue cryopreservation followed by autotransplantation has emerged as a promising strategy. This approach is particularly helpful for patients who require immediate cancer treatment and cannot undergo ovarian stimulation for oocyte or embryo cryopreservation. In this context, cortical fragments of ovarian tissue are surgically harvested and cryopreserved, with the goal of reimplanting them after cancer remission to restore fertility and endocrine function [4,5]. However, this technique presents limitations for patients with hematological malignancies or cancers with high metastatic potential, such as leukemia, neuroblastoma, or Burkitt lymphoma, where there is a significant risk of reintroducing malignant cells via the graft [6]. For

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these patients, in vitro culture of ovarian tissue or isolated follicles offers a safer alternative, aiming to support complete folliculogenesis in a controlled environment and ultimately enable the retrieval of developmentally competent oocytes for use in assisted reproductive technologies [7,8].

The success of in vitro folliculogenesis relies heavily on the ability to replicate the ovary's intricate biochemical and biophysical environment [9,10]. A key component of this process is the culture medium, which must deliver the appropriate biological and chemical signals to support follicular growth, oocyte maturation, and cell–cell communication [11]. In the case of isolated preantral follicles, maintaining the three-dimensional (3D) structure is critical for preserving physiological interactions. To this end, biomaterials such as alginate, fibrin, and polyethylene glycol (PEG)-based hydrogels have been extensively investigated as encapsulation matrices, offering mechanical support and enabling nutrient diffusion [9]. Cells also play a vital role in folliculogenesis by secreting growth factors and cytokines that support follicular development [12]. In ovarian tissue culture, this function is naturally provided by the native cellular environment. For isolated follicles, various support cells such as ovarian stromal cells can be incorporated into the culture system to improve the growth of follicles [13]. In addition, dynamic culture systems incorporating mechanical stimuli or continuous medium flow are increasingly recognized for their ability to better mimic the in vivo ovarian environment [14]. These systems improve oxygen and nutrient delivery, facilitate waste removal, and can exert beneficial mechanical forces that influence follicle viability and maturation.

Additionally, each culture method can preferentially support specific stages of folliculogenesis. For instance, Xu et al. [15] encapsulated mouse secondary follicles in alginate; the follicles formed antral cavities by day 8, and yielded metaphase-II oocytes upon maturation. This 3D culture yielded fertilizable oocytes and ultimately healthy live-born pups. Such systems support rodent follicles through the full gonadotropin-dependent stage (antral) and produce competent oocytes. In humans, encapsulation of preantral follicles in hydrogels has supported development up to secondary stage [8]. To achieve full maturation, multistep culturing systems have been developed, enabling human preantral follicle to progress to normal metaphase II oocytes [7]. Similarly, culturing ovarian cortex fragments has been shown to promote the activation and growth of human primordial follicle [16].

This review explores the key environmental factors that influence in vitro folliculogenesis, including biochemical, biophysical, and cellular components, and highlights recent innovations in culture systems designed to recreate the ovarian niche and promote optimal follicular growth and maturation.

## 2. Follicle dynamics

Folliculogenesis is a highly coordinated and dynamic process that governs the growth and maturation of ovarian follicles, culminating either in ovulation or follicular atresia. This progression is fundamental to female reproductive health, as it ensures the selection and development of oocytes capable of fertilization. The concept of follicle dynamics encompasses the sequential structural, cellular, and hormonal changes that follicles undergo throughout their development. Accurately replicating these dynamic processes is essential for the success of in vitro folliculogenesis protocols [17]. Follicular development begins with the activation of primordial follicles, which represent the largest yet quiescent pool of follicles in the ovary. These follicles consist of a central oocyte arrested in prophase I of meiosis, surrounded by a single layer of flattened pre-granulosa cells and a surrounding basement membrane. Upon activation, granulosa cells undergo morphological transformation from squamous to cuboidal, begin to proliferate, and support the growing oocyte, which increases in diameter. This marks the transition to the primary follicle stage, characterized by continued oocyte enlargement, granulosa cell proliferation, and deposition of the zona pellucida, a specialized glycoprotein matrix critical for oocyte protection and fertilization. As follicles advance to the secondary stage, theca cells differentiate into the theca interna, which becomes vascularized and hormonally active, and the theca externa, which provides structural support. The formation of the antral cavity defines the antral stage, where granulosa cells organize into cumulus and mural subpopulations with specialized roles in oocyte nourishment and steroidogenesis. From this point, the actions of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) are pivotal, driving further maturation and estradiol production [18,19].

It is important to note that the timeline of folliculogenesis varies across species. In humans, the progression from primordial to preovulatory follicle may take several months, while in rodent models this process is significantly faster [20]. For example, in rats, primordial follicles typically develop into secondary follicles more than 30 days, followed by an additional 30 days for antral follicle formation and preovulatory development [21]. Such interspecies differences must be carefully considered when translating in vitro findings to human reproductive applications.

Additionally, although thousands of follicles initiate development, more than 99 % undergo atresia, a hormonally regulated, active process involving granulosa cell apoptosis, autophagy, and/or necrosis, at various developmental stages before reaching the preovulatory phase [22,23]. This highlights the critical need for in vitro systems to deliver survival cues that prevent premature degeneration and mimic the natural regulatory checkpoints of the ovary.

A thorough understanding of these physiological events is pivotal for the design of in vitro systems that support not only proper follicular development but also oocyte competence and hormonal function [24]. Mimicking the dynamic interactions of the ovarian microenvironment can contribute to more efficient fertility preservation strategies, and provide valuable models for studying ovarian physiology, endocrine regulation, and reproductive toxicology.

## 3. Biomaterials in ovarian tissue engineering

Ovarian tissue engineering (OTE) aims to replicate the ovarian microenvironment by encapsulating follicles and ovarian cells within three-dimensional (3D) scaffolds that support their survival, development, and function during in vitro culture. The selection of appropriate biomaterials is a critical step in scaffold design, as these materials must function as a temporary artificial extracellular matrix (ECM) capable of supporting cell infiltration, adhesion, proliferation, and differentiation. Essential criteria for biomaterials in OTE include biocompatibility, biodegradability, and sufficient porosity to permit nutrient diffusion and waste removal [9].

The native ovarian niche also features a complex biochemical ECM. Key components include fibrillar collagens, laminins, fibronectin, and proteoglycans such as hyaluronan [25,26]. These molecules provide adhesion ligands and bind growth factors that support granulosa-oocyte signaling. For example, the basal lamina of growing follicles contains type IV collagen and laminin, which anchor granulosa cells, while stromal hyaluronan modulates tissue hydration and diffusion. Age-related biochemical changes, such as decreased hyaluronan content and increased fibrillar collagen deposition [27], an reduce growth factor availability and disrupt cell-matrix interactions, ultimately impairing oocyte competence.

In engineered culture systems, biomaterials such as collagen or peptide-modified polymers can be functionalized with RGD or other motifs that engage integrins and serve as biochemical signaling cues [27–30]. Mimicking the natural follicular microenvironment within engineered constructs requires careful consideration of multiple factors beyond biomaterial selection, including scaffold composition, mechanical properties, and incorporated biochemical signals.

Selecting biomaterials with appropriate mechanical properties is critical for providing a supportive structural niche, as these

characteristics must closely resemble those of native ovarian tissue to maintain follicle integrity. Scaffold mechanics influence cellular behavior through mechanotransduction pathways, such as YAP/TAZ and PI3K/AKT, that regulate cell phenotype, gene expression, and ECM production [31–34]. For example, Rosales et al. [35] demonstrated that reduced matrix stiffness in hyaluronic acid–based hydrogels led to a lower nuclear-to-cytoplasmic ratio of Yes-associated protein (YAP). Cells sense stiffness via integrins and other mechanosensors, triggering downstream pathways like Hippo/YAP and PI3K/AKT, which govern proliferation, apoptosis, and differentiation. Follicles cultured in high-stiffness gels showed rapid downregulation of developmental genes and upregulation of inflammatory and ECM-remodeling genes within hours [27,36]. Notably, impaired folliculogenesis has been observed in polycystic ovary syndrome (PCOS), a condition associated with significantly increased ovarian tissue stiffness compared to healthy controls [37].

One of the core challenges in scaffold development is achieving an optimal balance between mechanical rigidity and elasticity. This balance is vital to preserve the spherical morphology of the follicle and accommodate its radial growth over time [9]. As follicles enlarge, for instance, when human secondary follicles grow from approximately 120 μm to over 400 μm in diameter during antral development, the surrounding biomaterial must withstand the resulting mechanical stress while continuing to support cell function and expansion [38]. If the matrix is too rigid, it may restrict follicular growth; if too soft, it may collapse or fail to provide essential mechanical cues. Additionally, the degradation kinetics of the scaffold must be carefully tuned to allow gradual matrix remodeling and follicle growth without compromising tissue integrity. Ideally, the scaffold should degrade in synchrony with follicle expansion to maintain structural support and preserve morphology until it is replaced by newly formed functional tissue [39,40].

Both natural and synthetic biomaterials have been explored to fabricate these scaffolds. Ideal materials should not only mimic the biomechanical properties of native ovarian tissue but also provide biochemical signals that facilitate cell attachment, migration, differentiation, and ECM remodeling [41]. To this end, biomaterials are increasingly functionalized with bioactive motifs, such as RGD peptides or ECM-derived proteins, to promote integrin-mediated signaling and enhance follicular viability. Furthermore, emerging technologies such as 3D bioprinting now enable the design of spatially defined, physiologically relevant scaffolds with controlled architecture and cell distribution, offering a promising platform for personalized ovarian constructs. The 3D architecture of scaffolds, including pore size, geometry, and fiber diameter, plays a critical role in shaping in vitro folliculogenesis. Macropores on the order of follicle diameters (150–300 μm) are essential to physically accommodate secondary and preantral follicles without deformation, while micropores (5–20 μm) facilitate somatic cell adhesion and nutrient exchange. For example, Di Berardino et al. [42] engineered electrospun PCL scaffolds with ~300 μm interconnected macropores, enabling multiple lamb preantral follicles to survive, develop to early antral stages, and exhibit robust estradiol production over 18 days of culture. Similarly, a patterned electrospun gelatin scaffold with ~350 μm inter-strut spacing preserved porcine follicle sphericity and viability for at least 10 days, demonstrating that precise inter-fiber distances help prevent mechanical flattening and support follicular architecture [28].

Pore geometry also influences stress distribution: murine follicles cultured in angled-pore designs (30° strut orientation) showed higher survival rates and maintained a rounded morphology, whereas those in straight (90°) pores experienced single-point contact, excessive focal stress, and increased atresia [29].

Achieving the right combination of mechanical, biochemical, and architectural properties is central to developing functional scaffolds that can reliably support in vitro folliculogenesis. Table 1 summarizes commonly used biomaterials in OTE, highlighting their advantages,

**Table 1**
Overview of natural and synthetic biomaterials used in OTE, highlighting their advantages, limitations, and current strategies to enhance their performance.

| Biomaterials in OTE | | Favorable points for OTE application | Potential areas of improvement | Current solutions |
|---|---|---|---|---|
| **Natural** | Alginate | Promoting preantral follicle development *in vitro* and supporting ovarian cell proliferation [44]; Ability to be used in bioprinting [45,46] | Lack of cell binding sites and being inert [9] | Combining with fibrin, HAA, and gelatin [47–49], or conjugating bioactive molecules like RGD [30] |
| | Fibrin | Supporting vascularization and preantral follicle survival [50]; Restoring hormone cyclicity [51] | Rapid degradation and intrinsic instability [52] | Combining with alginate and PEG [8,53,54] |
| | Collagen | The most prevalent protein in the human body, providing a conducive environment for secondary follicle maturation supporting cell attachment, and sex hormone production [55] | Insufficient mechanical properties [56] | Blending with alginate or chitosan [56] |
| | dECM | Maintaining the ovarian preantral follicle/cells survival rate [57,58] | Diminished mechanical properties [59] | Careful optimization of protocols to preserve ECM integrity while removing cellular components [60] |
| | Chitosan | Biocompatible, biodegradable, with tunable mechanical properties, supporting ovarian preantral follicle integrity [61] | Low viscoelastic properties [62] | Combining with silk [63] |
| | HAA | Supporting the preantral follicle growth and hormone secretion [64] | Week mechanical properties rapid degradation [65] | Cross-linking with PEG [65] or combining with alginate [64] |
| **Synthetic** | PEG | Restoring ovarian endocrine function [66], and supporting ECM remodeling and tissue regeneration [67] | Lack of bioactive sites [68] | Blending with bioactive polymers, like fibrin [8], or conjugating with RGD [69] |
| | PCL | Providing the mechanical support to preserve preantral follicle morphology [28] | Lack of bioactive sites and hydrophobicity [70] | Combining with gelatin [71], or patterning [42] |

Abbreviations: HAA, Hyaluronic acid; SFX-1, produced from N-isopropyl acrylamide monomer; PCL, poly (epsilon-caprolactone); PEG, poly (ethylene glycol); dECM, decellularized extracellular matrix; RGD, arginine-glycine-aspartic acid.

limitations, and the innovative strategies developed to overcome current challenges. Different biomaterial formulations have been shown to support specific stages of folliculogenesis in a species-dependent manner. For instance, alginate-based hydrogel scaffolds promote the development of mouse follicles from the primary to early antral stage and, when functionalized with ECM cues or RGD peptides, can support maturation to metaphase II. In contrast, in human ovarian tissue, scaffolds such as alginate combined with fibrin or protein/peptide-modified PEG typically support follicle growth from the primordial or primary stage to the secondary stage. These differences highlight the need to tailor biomaterial composition, mechanical properties, and biochemical signaling to the developmental stage and species origin of the follicles [8,9,43].

### 3.1. Natural biomaterials in OTE

Natural biomaterials such as alginate, fibrin, and collagen offer significant potential for OTE due to their inherent biocompatibility, biodegradability, and resemblance to components of the native ECM. Among these, alginate is the most extensively used polymer for the in vitro culture of isolated preantral [72] and secondary [73,74] follicles, primarily because of its excellent biocompatibility, mild gelation conditions, and ability to form hydrogels under physiological conditions [9]. The mechanical and structural properties of alginate gels are modulated by factors such as molecular weight, degree of crosslinking, and the ratio of mannuronic to guluronic acid residues in its polymeric backbone [75].

Although alginate effectively supports early follicular development in vitro and provides a stable 3D environment, its bioinert nature, including the lack of natural cell adhesion sites, can impede follicle maturation and communication with the surrounding matrix [30]. To overcome this limitation, alginate has been chemically modified through the incorporation of the tripeptide arginine–glycine–aspartic acid (RGD), which facilitates integrin-mediated cell attachment and improves follicle–matrix interactions [43]. Alternatively, composite hydrogels that combine alginate with other natural ECM components, such as gelatin, collagen, fibrin, or hyaluronic acid, have been explored to better recapitulate the ovarian microenvironment and support the full progression of folliculogenesis [47–49].

Fibrin, another promising natural biomaterial, promotes cell adhesion and supports tissue remodeling, making it valuable for both in vitro primary and secondary follicle culture [76] and in vivo preantral and secondary follicle transplantation [77,78]. Its mechanical properties are tunable by adjusting fibrinogen and thrombin concentrations, allowing fine control over scaffold stiffness and degradation rate to match follicle growth requirements [79]. However, fibrin is prone to rapid degradation and has limited structural stability, which may compromise long-term culture systems [52]. To address this, strategies such as blending with alginate or crosslinking with polyethylene glycol (PEG) have been employed to prolong its lifespan and enhance its mechanical integrity [8,53,54].

Collagen, the main structural protein of the ovarian ECM [26], offers intrinsic bioactivity, including cell adhesion motifs and remodeling potential. It has been used as a 3D structure to support preantral follicle growth in vivo [80]. However, native collagen gels often lack sufficient mechanical strength and degrade quickly under culture conditions [81]. Therefore, combining collagen with other biomaterials or crosslinking it enzymatically or chemically could improve its performance in OTE applications.

The ECM of ovarian tissue forms a complex and dynamic network composed primarily of structural proteins and glycoproteins such as collagen, fibronectin, and laminin. This matrix not only provides physical scaffolding but also plays a critical role in regulating cellular behavior through biochemical signaling and mechanotransduction. In recent years, decellularized extracellular matrix (dECM) derived from ovarian or other supportive tissues has emerged as a highly promising biomaterial for follicle encapsulation and transplantation. By preserving native ECM architecture and biochemical cues, dECM can provide a more physiologically relevant environment for folliculogenesis [82,83]. Various decellularization protocols, including the use of non-ionic detergents (e.g., Triton X-100), ionic detergents (e.g., sodium dodecyl sulfate, SDS), and enzymatic agents (e.g., DNase I), have been employed to remove cellular components while retaining ECM integrity. Optimized combinations of these agents have been shown to enhance tissue compatibility, minimize immunogenicity, and promote hormone production and follicular survival following transplantation [84]. However, challenges remain in standardizing decellularization methods and improving the mechanical stability of dECM constructs, which often suffer from rapid degradation and inconsistent structural support.

To address these limitations, strategies such as crosslinking, blending dECM with more stable materials like alginate, or formulating dECM-based hydrogels have been employed. These approaches have demonstrated enhanced preantral follicle viability, improved antral formation, and support for oocyte maturation in vitro [85–87]. Moreover, the integration of dECM-derived bioinks into 3D bioprinting platforms has enabled spatial control over tissue architecture and enhanced vascularization and graft survival, pushing the boundaries of engineered ovarian constructs [88].

While natural biomaterials, including dECM, offer distinct advantages for OTE, continued refinement in their composition, processing, and mechanical performance is essential to fully meet the complex demands of supporting long-term follicle growth, hormonal function, and eventual oocyte competence in vitro and in vivo. Moreover, it is important to note that dECM-based materials are subject to batch-to-batch variation, as differences in tissue source and decellularization methods can lead to variability in composition and mechanical properties, posing a challenge for standardization and reproducibility in follicle culture applications.

### 3.2. Synthetic biomaterials in OTE

Synthetic biomaterials play an increasingly important role in OTE by offering highly customizable and reproducible scaffolds capable of supporting follicle growth, oocyte maturation, and tissue regeneration. Unlike natural materials, synthetic polymers allow precise tuning of mechanical, structural, and chemical properties, enabling the independent control of physical characteristics without compromising biochemical functionality. This decoupling of mechanical and biological traits represents a significant advantage over natural matrices, where alterations in composition (e.g., collagen or alginate concentration) often affect both stiffness and bioactivity simultaneously [89].

Among synthetic materials, PEG is one of the most widely studied polymers in regenerative medicine due to its excellent biocompatibility, low immunogenicity, and versatility. In its unmodified form, PEG is biologically inert; however, it can be functionalized with bioactive motifs, such as arginine–glycine–aspartic acid (RGD) peptides, to promote cell adhesion, and crosslinked with protease-sensitive peptides to allow matrix remodeling and cell migration. These modifications have enabled PEG-based hydrogels to support the viability, proliferation, and early development of primordial and primary follicles in preclinical models, while also facilitating angiogenesis and tissue integration, both essential for post-transplantation success [69].

More sophisticated formulations, such as 8-arm PEG-vinyl sulfone (PEG-VS), incorporate matrix metalloproteinase (MMP)- and plasmin-sensitive crosslinkers that closely mimic the dynamic behavior of the ovarian ECM. When used in conjunction with adipose-derived stem cells (ADSCs), these PEG-based systems promote primary follicle survival and development by preserving ADSC multipotency and stimulating the release of paracrine factors such as vascular endothelial growth factor (VEGF) and hepatocyte growth factor (HGF), which contribute to tissue regeneration and neovascularization [90].

Another promising synthetic material is polycaprolactone (PCL), a

biodegradable, FDA-approved polyester that offers good mechanical strength and slow degradation rates, making it suitable for longer-term culture systems. When blended with gelatin to form electrospun 3D fibrous scaffolds, PCL enhances follicle adhesion, nutrient exchange, and hormone secretion, and provides topographical cues that resemble the natural ovarian stroma [28].

Synthetic biomaterials are also increasingly being used in hybrid scaffolds that combine the mechanical precision of engineered polymers with the biological complexity of natural ECM components. These hybrid systems aim to harness the best of both worlds: the tunability and stability of synthetic scaffolds and the bioactivity and cellular responsiveness of natural matrices.

Through innovations in chemical modification, crosslinking, and scaffold architecture, synthetic biomaterials offer an adaptable and scalable platform for engineering ovarian tissues. Their inherent versatility positions them as powerful tools for advancing fertility preservation strategies, reproductive disease modeling, and ultimately, the clinical translation of artificial ovaries [91].

## 4. Culture medium and supplementations

The successful in vitro culture of ovarian follicles requires a carefully tailored culture environment that closely mimics the in vivo ovarian milieu. Central to this is the selection of an appropriate base medium and the strategic supplementation of nutrients, hormones, and signaling molecules to support follicular viability, growth, and oocyte competence (Fig. 1).

Commonly used basal media include Minimal Essential Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), Earle's Balanced Salt Solution (EBSS), and McCoy's 5 A medium, all of which provide essential ions, buffering capacity, and basic nutrients [92]. However, these media alone are insufficient for sustaining follicular development and must be enriched with specific additives to replicate the biochemical complexity of the ovarian microenvironment.

Energy substrates and metabolic support are fundamental. Glucose serves as a primary energy source for both somatic and germ cells, while L-glutamine and amino acids derived from fetuin support protein synthesis and cellular proliferation [93,94]. Ascorbic acid (vitamin C) plays a dual role by acting as an antioxidant and stabilizing the extracellular matrix, thereby reducing apoptosis and preserving follicular architecture [95]. Antibiotics are routinely added to prevent microbial contamination, particularly in long-term culture settings.

Insulin, transferrin, and selenium (ITS) are widely used to boost amino acid uptake and stimulate cellular metabolism, while also contributing to oxidative stress resistance [96]. In addition, protein supplements such as fetal calf serum (FCS), fetal bovine serum (FBS), and bovine serum albumin (BSA) provide growth factors, hormones, binding proteins, and lipids essential for cellular viability and oocyte maturation. Nonetheless, due to variability in serum composition, serum-free or defined media are increasingly preferred for reproducibility and clinical translation.

Hormonal regulation is another critical component. Supplementation with FSH and LH is essential for mimicking endocrine signals that regulate granulosa cell proliferation, steroidogenesis, and oocyte maturation. Human chorionic gonadotropin (hCG) is occasionally included to activate the LH receptor and promote final follicle maturation and ovulatory events [97]. However, optimal concentrations and timing are key. Excessive FSH, for example, has been associated with reduced follicle survival and impaired oocyte development [98]. In human preantral follicles, the addition of activin A has been shown to increase follicle diameter and enhance oocyte quality by modulating FSH receptor expression and granulosa cell proliferation [99,100].

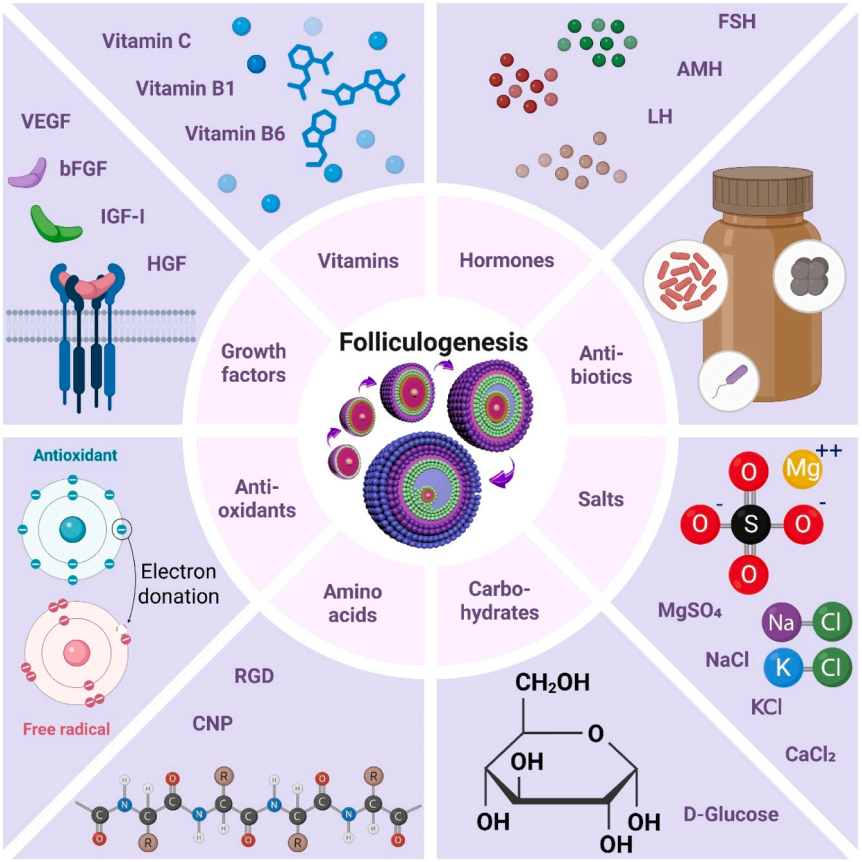


**Fig. 1.** Key biofactors in culture media influencing folliculogenesis.

Growth factors further refine the culture system. Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) play roles in somatic cell survival, angiogenesis, and tissue remodeling. Insulin-like growth factor I (IGF-I) enhances granulosa cell responsiveness to FSH and supports oocyte maturation [101]. More recently, emerging molecules such as kisspeptin and melatonin have demonstrated promising results in enhancing follicular activation, improving oocyte integrity, and reducing oxidative stress during culture [102,103].

The dynamic and multifactorial nature of folliculogenesis necessitates ongoing exploration of culture media compositions and supplement combinations. Table 2 provides an overview of recently employed additives and their functional roles in optimizing in vitro follicle culture systems. Continued refinement of these culture conditions is essential for advancing assisted reproductive technologies, with implications for both clinical fertility preservation and fundamental reproductive biology in human and veterinary settings.

Together, the materials and supplements discussed in this section highlight the critical importance of stage-specific optimization in in vitro follicle culture systems. Given the complexity and tightly regulated nature of folliculogenesis, continuous refinement of culture media and supplement combinations is necessary to support follicle growth and oocyte maturation at each developmental stage. Emerging molecules such as mTOR activators and Hippo pathway modulators [104] show promise in promoting primordial follicle activation and enhancing oocyte cytoplasmic quality. In later stages, hormonal supplements like FSH and LH, as well as growth factors such as EGF and IGF, have been effective in stimulating granulosa cell proliferation, antrum formation, and oocyte maturation [99–101]. These findings underscore the need to tailor culture conditions to the specific biological requirements of each follicular stage in order to enhance developmental competence and better recapitulate in vivo ovarian physiology.

## 5. Static cultivation

Static cultivation systems represent the foundational approach in ovarian follicle culture, providing a controlled environment without active fluid flow or mechanical stimulation. These systems have been instrumental in advancing our understanding of follicle development and in developing fertility preservation strategies. Depending on the experimental goals and follicle source, static culture can be applied to either individually isolated follicles or small tissue fragments, with various configurations designed to support growth, survival, and oocyte maturation under simplified yet physiologically relevant conditions.

**Table 2**
Overview of additives used in *in vitro* culture for follicle survival and maturation over the past decade.

| Additive | Concentration | Duration | Type of sample | Species | Effect |
|---|---|---|---|---|---|
| Insulin | 1 and 10 μg /ml | 14 days | SFs | Cat | Promoted follicle growth and survival, and antrum formation [105] |
| Combination of: FSH<br>Insulin<br>GH | 100 ng/ml<br>10 μg/ml<br>50 ng/ml | 18 days | SFs | Caprine | Enhanced preantral follicle follicle growth and oocyte meiotic resumption [106] |
| Human VEGFA 165 / bovine fetuin or both | 100 ng/10 mg | 6 days | Cortical pieces | Human | Reduced percentage of resting follicles and increased number of secondary growing follicles [94] |
| bFGF | 200 ng/ml | 8 days | Early follicles encapsulated into alginate | Human | Promoted development of preantral follicles [107] |
| GH/IGF−1 | 50 ng/ml GH | 1, 7 and 14 days | Tissue fragments | Bovine | GH → IGF−1 improved the development of preantral follicles until the first half of the culture [108] |
| LIF<br>IGF1<br>FGF2 | 2 μl/ml<br>20 ng/ml<br>40 ng/ml | 44 h | Fully-grown and growing oocytes | Pig | Enhanced the meiotic and developmental competence of oocytes [109] |
| EGF | 100 ng/ml | 2 days | Ovarian fragments | Equine | Promoted development of preantral follicles [110] |
| GDF−9 | 100 ng/ml | 12 days | SFs | Sheep | Improved growth, mitochondrial function, and oocytes meiotic resumption [111] |
| Growth hormone and FSH | 50 ng/ml | 18 days | PFs | Canine | Enhanced *in vitro* growth of secondary follicles [112] |
| BMP−15 | 15 ng/ml | 12 days | SFs encapsulated in alginate | Mouse | Improved production of mature oocytes [113] |
| BMP15 and FSH | 10 ng/ml and 100 mIU/ml | 7 days | Ovaries | Mouse | Increased percentage of antral follicles and ovarian size [114] |
| CNP | 100 nM/ml | 6 days | PFs | Murine | Enhanced oocyte's maturity [115] |
| Melatonin | $10^{-7}$ M | 18 days | SFs | Bovine | Increased diameter and rates of antrum formation [102] |
| Kisspeptin | 35 or 40 pmol | 8 days | Ovarian tissue | Human | Increased the expression of maturity genes (GDF9, BMP15 and mTOR) [104] |
| Kisspeptin | 10 μg/ml | 1 day | COCs | Sheep | Increased oocyte maturation [116] |
| Kisspeptin | 10 μg/ml | 6 days | PFs | Sheep | Resulted in better preantral follicle development [117] |
| Alpha-lipoic acid | 100 ng/ml | 6 days | Fragments of the ovarian cortex in agarose gel | Bos taurus indicus | Maintained follicular integrity [118] |
| Recombinant adiponectin | 5 and 10 μg/ml | 27 h | COCs | Goat | Enhanced the progression of goat oocyte nuclear maturation [119] |
| Q10 | 50 μM | 12 days | PFs | Mouse | Increased the diameter of preantral follicles, survival rate, antrum formation, and metaphase II oocytes [120] |
| Ascorbic acid | 100 ng/ml | 6 days | PFs on agarose matrix gel | Bovine | Promoted better conditions for preantral follicle development [121] |
| TNF-α | 10 ng/ml | 18 days | SFs | Bovine | Promoted growth and antrum formation, and maintained their viability [122] |
| Leucine, glutamine, and arginine | 200 μmol/l | 11 days | Ovaries | Murine | Accelerated the activation of primordial follicles [123] |
| Melatonin | 10 pM | 7 days | PFs form prepubertal ovaries | Mouse | Increased preantral follicle survival and diameter [124] |
| eCG | 0.05 IU/ml | 12 days | Multi-layered secondary and antral follicles | Cat | Stimulated growth of multi-layered secondary and early antral follicles [125] |

bFGF: Basic fibroblast growth factor, BMP15: Bone morphogenetic protein-15, CNP: C-type natriuretic peptide, COCs: Cumulus–oophorus complex, eCG: Equine chorionic gonadotropin, EGF: Epidermal growth factor, FGF2: Fibroblast growth factor 2, FSH: Follicle-stimulating hormone, GDF-9: Growth differentiation factor 9, GH: Growth hormone, IGF-1: Insulin-like growth factor 1, LIF: Leukemia inhibitory factor, PFs: Preantral follicles, SFs: Secondary follicles, TNF-α: Tumor necrosis factor alpha, VEGFA: Vascular endothelial growth factor A.

### 5.1. Isolated follicle culture

The in vitro culture of isolated ovarian follicles has undergone substantial progress in recent years, providing not only a powerful tool to study the fundamental biology of folliculogenesis but also a promising avenue for clinical and conservation applications [126]. By allowing direct observation and manipulation of isolated follicles, this approach enables researchers to explore oocyte–somatic cell interactions, hormonal responsiveness, and the influence of extracellular environments under controlled conditions.

Beyond its scientific value, isolated follicle culture holds significant translational potential in fertility preservation, particularly for patients at high risk of ovarian damage due to gonadotoxic cancer therapies, genetic disorders, or autoimmune diseases. In such cases, isolating and growing follicles in vitro circumvents the risk of reintroducing malignant cells associated with ovarian tissue transplantation. Additionally, this strategy is being explored in the field of wildlife conservation, where preserving and developing follicles from endangered species may offer a novel approach to maintaining biodiversity [127].

To date, a wide range of two-dimensional (2D) and three-dimensional (3D) culture systems have been developed to support the growth of isolated follicles in vitro (Fig. 2). These platforms have yielded encouraging results in both human and non-human mammalian models, with some systems supporting the progression from preantral to antral stages, and even the generation of developmentally competent oocytes in animal studies [128,129].

However, the complexity of folliculogenesis, which involves tightly regulated interactions between the oocyte, granulosa cells, theca cells, and the surrounding ECM, remains a major challenge for in vitro culture systems. Reproducing these physiological cues requires a fine balance of biochemical, mechanical, and spatial factors, making it difficult to optimize culture conditions across different follicle stages and species [130].

Within this context, the choice between individual versus group culture systems continues to be debated. Individual follicle culture allows for precise environmental control, detailed monitoring of developmental progression, and minimized paracrine interference from neighboring follicles. However, it may lack the physiological relevance of group cultures, where inter-follicular communication can influence development through shared signals and resource exchange [127]. Thus, the design of an optimal culture strategy depends on the specific goals of the study, whether focused on mechanistic understanding, oocyte yield, or translational applicability.

The culture of individual follicles offers the advantage of precise control over the microenvironment, enabling detailed monitoring of follicular development, survival, and oocyte quality. This system is particularly useful for dissecting the role of specific factors or conditions affecting isolated follicles. For instance, Guo et al. [131] demonstrated that supplementing individual secondary follicle cultures with 100 ng/ml neurotrophin-4 (NT4) significantly enhanced follicular growth, steroid hormone production, and oocyte maturation, yielding a higher proportion of metaphase II oocytes (43.5 % vs. 16.7 %) and leading to fertilizable blastocysts.

Despite these advantages, the isolation of early-stage follicles, particularly primary and preantral follicles, remains technically challenging. These follicles are structurally fragile, and the physical separation process often disrupts critical interactions between the oocyte and its surrounding granulosa cells, interactions essential for proper development and oocyte competence. As noted by Hornick et al. [132], individual culture systems often fail to sustain the development of primary murine follicles, resulting in increased rates of follicular atresia. Similarly, in a study comparing individual and group cultures of human secondary follicles, only those cultured in co-culture conditions progressed to oocyte maturation, while individually cultured follicles failed to mature [133].

By contrast, group culture systems more closely replicate the native ovarian microenvironment, primarily through the preservation of paracrine signaling and interfollicular communication. These interactions are believed to enhance follicular growth, steroidogenesis, and oocyte maturation. Hornick et al. [132] showed that co-cultured murine primary follicles secreted higher levels of growth factors and displayed increased formation of transzonal projections, which facilitated granulosa cell proliferation and supported oocyte competence. Similarly, co-cultured murine follicles demonstrated improved antral formation and a higher proportion of fertilizable oocytes compared to individually cultured counterparts [134].

However, group culture is not without drawbacks, particularly in mono-ovulatory species such as humans, cows, and sheep. In these species, typically only one dominant follicle reaches full maturation and ovulates, while subordinate follicles are suppressed through inhibitory endocrine and paracrine signals, ultimately undergoing atresia via apoptosis [135,136]. This physiological competition may limit the

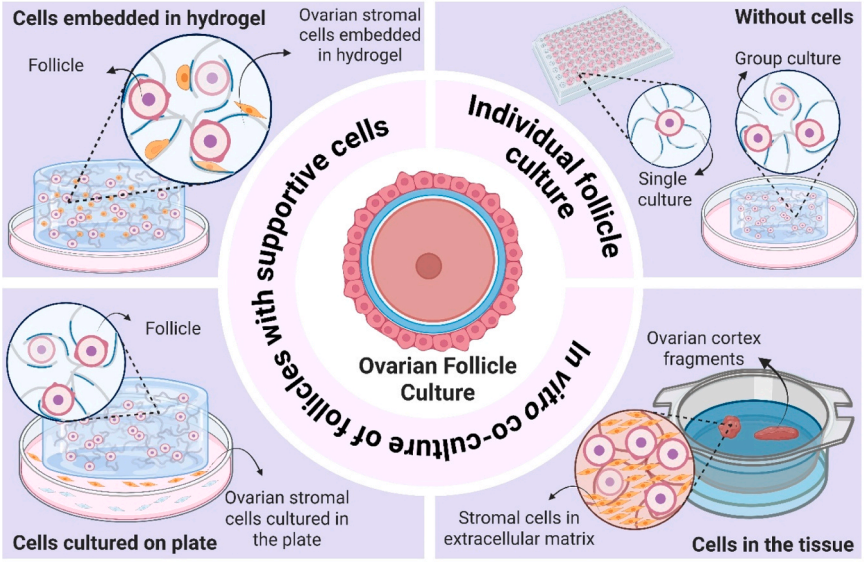


**Fig. 2.** Static cultivation of ovarian follicles, including individual follicle culture without supporting cells and in vitro culture with cellular support, such as feeder cells, hydrogel embedding, and ovarian tissue culture approaches.

success of group culture in these models. For example, Sirotkin et al. [137] reported that co-culturing bovine follicles led to a reduction in the secretion of insulin-like growth factor I (IGF-I), progesterone, and androstenedione, likely reflecting early atresia in subordinate follicles. Since IGF-I is known to inhibit follicular apoptosis and support steroidogenesis [138], its reduction may signal a paracrine suppression mechanism that mirrors in vivo dominance hierarchies.

The success of isolated follicle culture systems largely depends on the species and the developmental stage of the starting follicle. Group culture systems have demonstrated clear benefits for early-stage murine and intermediate-stage porcine follicles, where paracrine interactions among neighboring follicles enhance survival, proliferation, and structural integrity [132,134]. In contrast, individually cultured secondary or unilaminar follicles, especially in human models, have progressed successfully to the antral stage and even to metaphase II under optimized multi-step or matrix-free 3D protocols [129,131,133].

Therefore, the choice between individual versus group follicle culture should be guided by the specific goals of the study or clinical application. Individual culture systems provide precise environmental control and allow mechanistic studies at the single-follicle level, making them ideal for developmental and toxicological research. In contrast, group culture systems better mimic the in vivo follicular niche, enabling collective signaling and potentially enhancing developmental competence, particularly when multiple follicles are needed for oocyte retrieval or tissue engineering applications.

### 5.2. *In vitro co-culture of follicles with supportive cells*

While ovarian tissue culture preserves the native microenvironment and supports early stages of folliculogenesis, it still faces limitations in fully recapitulating the dynamic cellular signaling required for complete oocyte maturation. To address this, researchers have explored co-culturing isolated follicles with specific supportive cell types, particularly stem cells, as a means to more precisely modulate the biochemical and structural cues that drive follicle development. These cell-based systems enable targeted manipulation of the follicular niche, opening new avenues for enhancing survival, activation, and functional maturation of follicles in vitro.

#### 5.2.1. *Co-culture of isolated follicles with supportive cells*

Co-culturing isolated ovarian follicles with supportive cell types, particularly stem cells (SCs), has emerged as a promising strategy to enhance follicular growth and development by better replicating the complex paracrine and cellular interactions of the native ovarian environment. This approach leverages the trophic support, secretory profile, and regenerative potential of stem cells to create a more physiological microenvironment.

Among various stem cell types, human mesenchymal stem cells (MSCs) have shown particularly strong benefits. In addition to their differentiation potential and role in tissue repair [139], MSCs exert potent paracrine effects, modulating angiogenesis, anti-apoptotic signaling, and immune responses [139,140]. Their secretome, rich in growth factors, cytokines, and ECM components, has been shown to promote follicle survival, early activation, and continued development [141,142]. Co-culture with MSCs has demonstrated improved viability and activation of preantral follicles, primarily through local release of supportive factors that mimic stromal niche interactions [124,125].

Human menstrual blood-derived stem cells (MeSCs) also represent a clinically appealing and non-invasive source of support cells. MeSCs have been reported to enhance follicular growth, increase survival rates, and upregulate oocyte-specific genes such as *Bmp15* and *Gdf9*, which are essential for oocyte competence and folliculogenesis [141]. Additionally, endometrial MSCs derived from menstrual blood have been shown to promote germline stem cell renewal, follicle viability, and potentially restore ovarian function in models of premature ovarian insufficiency (POI) [143,144].

The integration of three-dimensional (3D) scaffolds with stem cell co-cultures has further improved outcomes by recreating the spatial architecture and mechanical support of the ovarian ECM. These biomaterial-based matrices facilitate bidirectional signaling between follicles and supportive cells, which is critical for proper development. For instance, encapsulation of follicles with MeSCs in alginate beads yielded greater follicle viability compared to collagen-based matrices, suggesting that both the type of co-cultured supportive cells and the choice of matrix significantly influence follicular outcomes [141].

Similarly, co-culturing goat preantral follicles with MSCs in FBS-free medium promoted early activation and growth, improving both survival and follicular diameter, while minimizing variability introduced by undefined serum components [142]. Novel scaffold systems such as decellularized mesenteric peritoneal membrane (DMPM) or co-culture with peritoneal mesothelial stem cells (PMSCs) have demonstrated differential but complementary effects: DMPM promoted follicle expansion, whereas PMSCs supported higher follicular viability and estradiol production, contributing to a more functional endocrine microenvironment [145]. This combination of a scaffold and MSC-derived bioactive molecules creates a natural-like ECM structure, allowing bidirectional signaling between the oocyte and somatic cells essential for folliculogenesis [146].

Notably, PEG-based 3D scaffolds loaded with adipose-derived stem cells (ADSCs) have also shown strong synergistic effects. This model supported increased follicle survival and growth, as well as enhanced expression of angiogenesis-related genes such as *VEGFA*, *HGF*, and *TGFB* [147]. Moreover, PEG hydrogels preserved ADSC multipotency more effectively than 2D systems, as demonstrated by upregulation of stemness-associated genes including *NANOG* and *SNAI2* [147].

Taken together, these findings underscore the importance of combining stem cell support with biomimetic 3D scaffolds to optimize the in vitro culture of isolated follicles. This integrated approach facilitates essential paracrine and mechanical signaling, supports cell survival and growth, and maintains gene expression profiles relevant to folliculogenesis, multipotency, and oocyte maturation. Notably, the effectiveness of these strategies is stage-dependent. Stem cell–based systems are particularly advantageous for primordial and primary follicles, promoting their activation and survival. In contrast, scaffold-integrated co-cultures yield improved outcomes for secondary and antral follicles, enhancing growth, steroidogenesis, and oocyte competence. As the field advances, such co-culture models offer exciting prospects for fertility preservation, reproductive toxicity testing, and the development of artificial ovaries.

#### 5.2.2. *Tissue cultures*

Folliculogenesis, the cornerstone of female reproduction, naturally takes place in the pubertal ovary and involves the activation of dormant primordial follicles followed by their growth and development toward the preovulatory stage. To obtain mature oocytes suitable for in vitro fertilization (IVF), two main strategies are currently available: ovarian tissue transplantation and in vitro folliculogenesis. However, in patients at risk of malignant cell reintroduction, such as those with leukemia or other hematologic malignancies, ovarian tissue transplantation following cryopreservation is contraindicated [148]. Moreover, even in safe candidates, transplantation is typically associated with a high rate of follicular loss, which limits the optimal use of the primordial follicle pool [149].

In this context, in vitro culture of ovarian tissue represents a promising alternative. This approach preserves the native ovarian microenvironment, including the presence of theca cells and components of the extracellular matrix (ECM), both of which are critical for supporting follicular development and steroidogenesis [150]. By maintaining this physiologic niche, ovarian tissue culture provides structural and biochemical cues that are often missing in isolated follicle systems.

In vitro folliculogenesis using tissue culture is a multi-step and stage-specific process [129]. In mice, the first live birth from an oocyte fully

grown in vitro was achieved in 1996 [151]. In that study, whole neonatal ovaries were cultured in vitro for 8 days, after which oocyte–granulosa cell complexes were isolated and further cultured for 14 days [151]. This sequential approach successfully yielded fertilizable, developmentally competent oocytes, and marked a breakthrough in achieving in vitro follicle activation, the critical first step in the culture of primordial follicles.

In human ovarian tissue, follicle activation can be stimulated using PI3K activators or PTEN inhibitors, targeting pathways involved in early folliculogenesis [152]. In vitro maturation of primordial follicles to the secondary stage has been demonstrated after 8 days of culture in FSH-enriched media, and up to 36.7 % of primordial follicles can progress to the antral stage under optimized conditions. Notably, polar body-containing oocytes have been obtained following in vitro maturation (IVM) after 21 days of culture, supporting the feasibility of multi-stage follicular development in vitro [129].

Alternative approaches to improve follicle survival and minimize premature activation include xenografting ovarian tissue onto the chorioallantoic membrane (CAM) of avian embryos. This method facilitates rapid revascularization, reducing ischemic stress and offering a protective effect on the ovarian reserve [153,154]. CAM culture has been shown to preserve more quiescent follicles compared to conventional in vitro culture. However, ethical considerations arise due to inevitable integration of avian blood cells within the grafted tissue, raising concerns for clinical application.

Endocrine background significantly influences the success of ovarian tissue culture. Tissue obtained from adult trans masculine donors (following testosterone treatment) do not provide high-quality follicles if compared to ovaries from cis female donors [155]. The endocrine changes from the pubertal age to adulthood reduce the capacity of follicles to develop *in vitro* [156]. Despite their larger availability, follicles included in prepubertal tissue show a reduced capacity of activation and development *in vitro* [157]. Interestingly, pharmacological inhibition of estrogen receptors has been shown to increase the transition from primordial to secondary follicles in immature ovarian tissue, offering new avenues for enhancing developmental competence [157].

An additional factor to consider is the impact of cryopreservation prior to in vitro culture. Although ovarian tissue cryopreservation techniques are increasingly standardized and widely adopted, the freezing and thawing process may still compromise cell viability, tissue morphology, and ECM integrity, potentially affecting follicular outcomes.

In summary, ovarian tissue culture systems have now been shown to support folliculogenesis across multiple stages, from primordial follicle activation to preantral and, in some protocols, full progression into early antral and even metaphase II oocytes.

### 5.3. Reconstituted follicle systems

Emerging reconstituted follicle systems have demonstrated that the entire ovarian developmental program can now be reconstructed in vitro. For instance, Morohaku et al. [157] combined mouse primordial germ cells (PGCs) with gonadal somatic cells to reconstruct a functional ovary in culture. Their system recapitulated meiosis and oocyte growth, ultimately producing mature oocytes capable of fertilization and generating healthy offspring. El-Hayek et al. [158] further showed that the growing oocyte actively remodels its somatic microenvironment: oocyte-secreted GDF9 induces granulosa cells to form transzonal projections (TZPs) through the zona pellucida, thereby amplifying germline–somatic signaling. Notably, they observed a marked reduction in TZPs in aged mouse follicles, suggesting that impaired oocyte–granulosa communication may contribute to age-related fertility decline. Similarly, Fushii et al. [159] demonstrated that even denuded bovine oocytes can re-establish TZP connections and resume normal growth when co-cultured with mural granulosa cells.

More recently, Granados-Aparici et al. [160] reported that deletion of SMAD4, the canonical TGFβ signaling mediator, resulted in a 20–50 % reduction in TZP numbers and a 50 % downregulation of the transmembrane proteins N-cadherin and Notch2, both crucial for cell-cell communication during folliculogenesis. Complementary findings by Wang et al. [161] showed that transferring aged oocytes into a young follicular microenvironment significantly improved meiotic maturation, blastocyst formation, and live birth rates compared to aged oocytes left in an aged environment.

Together, these studies underscore the utility of reconstituted follicle platforms not only for fertility preservation but also for dissecting the molecular dialogue between oocytes and granulosa cells that governs follicle development and contributes to reproductive aging.

## 6. Dynamic cultivation

While static culture systems have significantly advanced our understanding of folliculogenesis and supported early-stage oocyte development, they inherently lack key physiological features of the in vivo ovarian environment, namely continuous nutrient exchange, mechanical stimulation, and dynamic waste removal [162]. These limitations can impair long-term follicle viability, hinder antral formation, and reduce the efficiency of oocyte maturation [163,164].

To address these challenges, dynamic culture systems have been developed to introduce controlled fluid flow and biomechanical stimuli that more accurately replicate ovarian tissue physiology. Platforms such as perfusion bioreactors and ovary-on-a-chip (OVoC) technologies offer enhanced environmental control, allowing researchers to optimize follicular development, hormonal signaling, and tissue functionality under more physiologically relevant conditions. These innovations mark a significant step forward in reproductive tissue engineering and open new possibilities for both basic research and clinical applications.

### 6.1. Bioreactors

Bioreactors represent a transformative approach in the field of reproductive tissue culture, enabling the precise regulation of physical and biochemical parameters critical for folliculogenesis. By incorporating features such as perfusion flow, oxygenation, and mechanical stimulation, these systems provide a dynamic environment that more closely mimics the in vivo ovarian niche. Bioreactors can be adapted to support the culture of whole ovaries, tissue fragments, or isolated follicles, and are particularly effective in enhancing nutrient delivery, waste removal, and mechanotransduction, all critical for sustaining long-term follicular viability, development, and function. A variety of bioreactor configurations have been explored in ovarian tissue engineering, each offering distinct advantages and technical considerations depending on the target tissue and application.

In a pioneering study, Zanotelli et al. [164] developed a perfusion bioreactor to culture whole bovine ovaries via intravascular delivery of culture medium through the main ovarian artery. The system included a custom-designed chamber integrated with a peristaltic pump for controlled media flow. Two perfusion strategies were compared: continuous perfusion with fresh medium and recirculation of output medium. The latter led to medium acidification (pH ~6.27 vs. ~7.72) and a substantial increase in apoptotic cell death, approximately 8-fold after 24 h and 5-fold after 48 h, underscoring the importance of fresh medium supply to maintain tissue health and homeostasis [164].

Beyond nutrient and gas exchange, mechanical forces such as shear stress and compressive strain have emerged as key modulators of cellular behavior in tissue culture systems. These biomechanical cues promote cellular viability, ECM organization, and tissue remodeling. For instance, dynamic perfusion bioreactors have been successfully applied to tissue culture in many organ models. For example, in cartilage tissue engineering, perfusion bioreactors increased cartilage quality [165] by promoting extracellular matrix deposition and facilitate the uniform seeding of viable chondrocytes [166]. Similarly, vascular bioreactors

with oxygen perfusion and cyclic flow [167] or pulsative mechanical stretch [168] more accurately replicate the mechanical environment of the cardiovascular system, enhancing vascular cells' seeding. Similarly, the ovary is increasingly recognized as a mechanosensitive organ, where both mechanical inputs and cell signaling pathways are critical for normal development and folliculogenesis [169,170].

Building on this concept, Barbato et al. [163,171] developed an innovative dynamic perifusion bioreactor specifically for ovarian tissue culture. The device features a conical chamber divided into two halves, separated by a porous membrane that allows culture medium to flow perpendicularly across ovarian tissue strips. The tissue is held in place by polyester nets, ensuring stable positioning and uniform exposure to compressive forces and shear stress [163,171]. Compared to static systems, this dynamic setup significantly improved follicle quality, survival, and developmental progression. Notably, it increased the proportion of healthy secondary follicles in both bovine and human ovarian tissues and enhanced estradiol production, an indicator of endocrine function and follicular activity [163]. The detailed structure and operation of this perifusion bioreactor are shown in Fig. 3, which presents both an exploded view of its components and the assembled configuration of the full experimental setup. As bioreactor systems evolve, they hold potential not only for enhancing oocyte yield in fertility preservation protocols but also for use in preclinical toxicology and regenerative medicine as physiologically relevant in vitro ovarian models. Despite their promise, the widespread adoption of bioreactor systems remains limited by high costs, technical complexity, and the need for standardization to ensure reproducibility across laboratories. Emerging efforts aim to integrate bioreactor systems with real-time biosensing, omics technologies, and AI-driven feedback control to fine-tune the culture environment dynamically and personalize follicle development protocols.

Whole ovary perfusion systems help preserve global tissue viability, whereas localized perfusion systems enhance the quality and development of secondary follicles, making them particularly well suited for studies on folliculogenesis, hormone production, and fertility preservation. The integration of dynamic stimuli into ovarian culture systems represents a major step forward in recreating physiologically relevant in vitro environments. Bioreactor technology offers a scalable and customizable platform for improving follicle maturation and function, with direct applications in fertility preservation, toxicology, and reproductive research.

### 6.2. Ovary-on-a-chip

The organ-on-a-chip (OoC) platform integrates tissue engineering with microfluidic technologies to mimic essential features of living organs, including cell-cell interactions, extracellular microenvironments, and dynamic physiological responses. Originally developed as an alternative to animal testing, OoC systems are now used across a broad range of applications, from modeling healthy and diseased organs to studying developmental biology and accelerating drug discovery through preclinical testing of efficacy, toxicity, and target engagement [172–174].

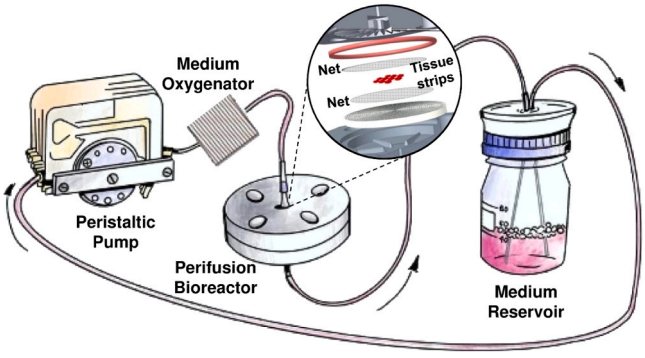


**Fig. 3.** Schematic of the bioreactor and experimental setup. Assembled configuration of the system for tissue culture, including the medium reservoir, medium oxygenator, peristaltic pump, and perfusion bioreactor, where the tissue strips were cultured between two nets [163].

To faithfully replicate the multifaceted environment of a target organ, OoC designs incorporate microchannel networks for precisely controlled fluid flow, cell chambers to host functional tissue units, and filters or barriers that regulate cell positioning and intercompartmental exchange [175–177]. Compared to conventional 3D static cultures, microfluidic platforms offer several advantages, including continuous nutrient and oxygen delivery, waste removal, and the ability to apply mechanical stimuli such as shear stress under tightly controlled conditions. Importantly, these systems can interconnect multiple tissues, enabling the investigation of inter-organ communication and systemic physiological responses [178]. Despite the rapid development of OoC systems for organs such as the liver liver [179], lung [180], vessel [181] and kidney [182], the ovary-on-a-chip (OVoC) remains a relatively new and evolving technology.

The application of OVoC to folliculogenesis is gaining momentum, although species-specific optimization remains a key area of ongoing research. For instance, Nagashima et al. [183] developed a microfluidic culture system to assess ovarian cortical tissue viability from domestic cats and dogs under flow rates of 2 and 10 μL/min. While cat tissues tolerated dynamic conditions well, dog follicles showed better outcomes in static culture, suggesting that flow parameters must be carefully tailored for each species.

In a separate study, human secondary follicles encapsulated in 0.5 % alginate hydrogels were cultured for 8–10 days under both static and dynamic conditions using an OVoC system operating at a flow rate of 8.33 μL/h. Although follicle diameter increased similarly in both conditions, estradiol secretion was significantly higher in the dynamic setting, indicating enhanced endocrine responsiveness without impairing follicular structure [184]. Xiao et al. [185] introduced the Solo-MFP OVoC platform, which enabled complete in vitro follicle maturation from the primary stage to metaphase II (MII) oocyte release and granulosa cell luteinization, under a flow rate of 40 μL/h. These findings underscore the feasibility of using OVoC systems to support the full trajectory of folliculogenesis, though further studies are needed to refine culture conditions across species and follicle stages.

Beyond follicle growth, OVoC platforms have proven especially valuable for modeling dynamic ovarian endocrine function. Won et al. [186] evaluated engineered bi-layered and tri-layered follicles with Matrigel interfaces, demonstrating preserved architecture and hormonal activity for up to 30 days of dynamic cultivation.

Further advancing the field, Xiao et al. [185] developed the Solo-MFP, Duet-MFP, and Quintet-MFP platforms, which integrate reproductive tissues such as the ovary, fallopian tube, uterus, and cervix, along with the liver, to replicate systemic hormonal cycles resembling the human menstrual cycle (Fig. 4). These platforms enable precise simulation of organ-organ communication and feedback loops, offering a robust experimental framework for exploring cyclic endocrine regulation and reproductive physiology. Ovary-on-a-chip platforms have been shown to support the development of early preantral and secondary follicles through to the antral stage. More advanced systems, such as the Solo-MFP platform, have further enabled complete follicle maturation, including metaphase II oocyte release and granulosa cell luteinization, under precisely controlled flow conditions.

In addition to folliculogenesis and endocrine research, OVoC technology has been applied to disease modeling and toxicological screening. These systems have been used to recreate ovarian cancer microenvironments [187–189]. The ability to mimic disease-relevant conditions while preserving physiological signaling makes OVoC a promising platform for personalized medicine and preclinical reproductive toxicology.

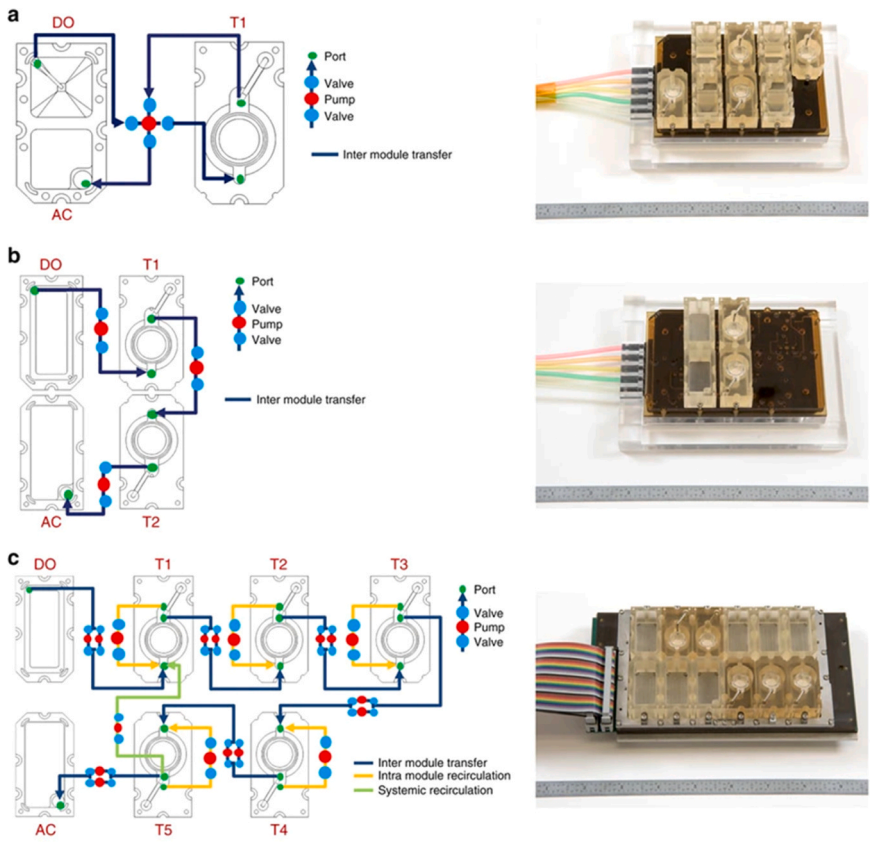


**Fig. 4.** Schematic diagrams and representative images of the microfluidic pumping platforms developed by Xiao et al. [185]. (a) The Solo-MFP configuration featuring a single integrated pump unit; (b) the Duet-MFP system, which employs a pair of coordinated pump modules for enhanced flow control; and (c) the Quintet-MFP architecture, comprising five interconnected pump units to enable complex fluid routing. Key functional compartments are designated as follows: AC, acceptor module; DO, donor module; T, tissue module.

Looking ahead, the potential of OVoC platforms can be further expanded through their integration into multi-organ systems, enabling the modeling of complex physiological interactions such as the hypothalamic–pituitary–ovarian axis. This would allow researchers to more accurately simulate systemic endocrine feedback and pharmacokinetics, particularly for drug testing or hormone-related disorders. The use of patient-derived cells, including those differentiated from induced pluripotent stem cells (iPSCs), also opens the door to personalized reproductive models, which may guide individualized treatment strategies for infertility or ovarian dysfunction. Nonetheless, key technical challenges remain, including the standardization of protocols, scalability, and cost-effective fabrication, all of which must be addressed to

**Table 3**
Comparative overview of in vitro follicle culture strategies.

| Culture approach | Advantages | Limitations | Applications | Future directions |
|---|---|---|---|---|
| Individual isolated follicle culture | Precise control of the microenvironment; useful for mechanistic studies; avoids inter-follicle paracrine interference | Technically challenging to isolate early-stage follicles; high atresia rates; limited physiological relevance | Basic research; toxicology; fertility preservation for cancer patients | Integration with dynamic systems; better automation for high-throughput screening; improved follicle retrieval techniques |
| Group of isolated follicles | Mimics paracrine communication; supports oocyte maturation; improved hormone secretion | Risk of dominance-related suppression in mono-ovulatory species; reduced individual control | Oocyte yield optimization; developmental competence studies | Development of species-specific group culture protocols; modulation of dominance signals; optimized media for synchronized growth |
| Co-culture with supportive cells | Enhances survival and growth via paracrine factors; can restore follicle competence; compatible with 3D matrices | Stem cell source and quality variability; requires optimization of scaffold and cell ratios | Artificial ovary development; regenerative medicine; preclinical ovarian models | Standardization of cell sources; scalable scaffold designs; exploration of gene-editing for enhanced paracrine signaling |
| Tissue culture | Maintains native ECM and cellular architecture; supports early follicle stages; no need for enzymatic digestion | Limited to early follicle stages in many species; tissue variability; cryodamage risk | Fertility restoration; early follicle activation; cryopreservation alternatives | Use of scaffold-supported tissue culture; improved cryopreservation recovery; hormonal modulation to boost maturation |
| Dynamic bioreactor systems | Improved nutrient and oxygen delivery; mechanical stimulation enhances growth and viability; dynamic waste removal | Complex setup; requires fresh medium supply; expensive equipment; scalability concerns | Advanced tissue engineering; translational studies; improving IVF efficiency | Miniaturization and cost reduction; real-time monitoring systems; integration with omics technologies |
| OVoC | Highly controlled microenvironment; supports multi-organ interactions; mimics endocrine loops; enables dynamic hormone regulation | Still in early stages for ovarian applications; technical complexity; species-specific responses; limited accessibility | Multi-tissue interaction modeling; endocrine function; drug testing and toxicology | Improved chip accessibility; broader validation across species; enhanced connectivity with other reproductive tissues |

facilitate broader adoption and clinical translation. Finally, the integration of real-time biosensors into OVoC systems could enable continuous monitoring of hormone levels, metabolic activity, and follicular health, significantly enhancing their value in both basic and translational research.

A comparative overview of the major follicle culture approaches, including their respective advantages, limitations, applications, and future directions, is summarized in Table 3.

## 7. Possible effects of *in vitro* culture on the oocytes

The in vitro culture environment, while essential for advancing fertility preservation strategies, may pose significant risks to germ cells, particularly oocytes. These risks are not limited to cell death but also encompass functional impairments and genetic or epigenetic alterations that can compromise follicular development and oocyte competence.

One of the primary concerns is oxidative stress, which arises from supraphysiological oxygen levels commonly used in vitro. Elevated $O_2$ tension leads to the accumulation of reactive oxygen species (ROS), which can damage lipids, proteins, and nucleic acids [190]. Granulosa cells, which play a key role in supporting the oocyte, are particularly sensitive to ROS via the ROS–JNK–p53 pathway, with hydrogen peroxide ($H_2O_2$) acting as a major mediator [191]. In oocytes, ROS production is further exacerbated by ischemic conditions that begin during tissue transportation and storage prior to culture. Conversely, hypoxic conditions during in vitro culture have been shown to alter genomic DNA methylation patterns, suggesting that both extremes of oxygen exposure can disrupt oocyte epigenetic integrity [192].

Another important consideration is the impact of in vitro conditions on mitochondrial function, which is vital for oocyte maturation, chromosomal segregation, and embryo development [193–196]. In vitro culture may disrupt mitochondrial distribution, reduce ATP production, and alter mitochondrial membrane potential, all of which can compromise spindle assembly and oocyte quality. In parallel, meiotic spindle integrity is particularly vulnerable to oxidative and thermal stress, with disruptions leading to chromosome misalignment and an increased risk of aneuploidy, compromising embryo viability.

The epigenetic landscape of the oocyte is particularly vulnerable during IVM. Several studies have reported alterations in DNA methylation, including both hypomethylation and hypermethylation, which may impact the expression of genes essential for early embryonic development [197,198]. Moreover, the mRNA expression profiles of developmentally important genes differ significantly between in vivo and in vitro matured oocytes [199], indicating that culture conditions can interfere with the regulation of gene expression critical for fertilization and embryogenesis.

Beyond biochemical stressors, mechanical manipulation and suboptimal culture conditions also threaten oocyte quality. Physical stress during follicle or oocyte isolation, exposure to inappropriate culture media, and environmental fluctuations can negatively influence developmental outcomes [200]. In particular, temperature variations [201] and exposure to light during handling [202] have been shown to reduce fertilization rates and impair blastocyst formation [203].

To mitigate some of these risks, research has explored the addition of antioxidants to the culture medium. Compounds such as melatonin [196], resveratrol [204], and N-acetylcysteine [205] have demonstrated protective effects against oxidative damage, improving mitochondrial function, and enhancing follicle quality during in vitro growth.

These insights highlight the necessity of optimizing in vitro culture conditions to more closely mimic the natural ovarian microenvironment. Reducing oxidative stress, maintaining epigenetic stability, and minimizing mechanical and environmental stressors are essential to preserve oocyte quality and developmental competence in vitro.

## 8. Developmental capacity and live birth outcomes

One key measure of success in in vitro folliculogenesis is the generation of oocytes that not only reach metaphase II (MII) but also support fertilization, embryogenesis, and live birth. To date, only rodent models have fully demonstrated this capacity under entirely in vitro conditions. In a landmark study, Eppig and O'Brien [151] cultured neonatal mouse ovarian tissue for eight days, allowing primordial follicles to develop to the preantral stage. The resulting oocyte–granulosa cell complexes were then cultured individually for 14 additional days, yielding MII oocytes that, after in vitro fertilization (IVF) and embryo transfer, produced healthy, fertile offspring. Building on this approach, Xu et al. [15] encapsulated mouse secondary follicles in an alginate hydrogel. After 8 days of culture, the follicles formed antral cavities, and the oocytes matured to MII. IVF of these oocytes generated viable embryos and resulted in fertile offspring of both sexes, demonstrating that a synthetic 3D microenvironment can recapitulate the in vivo niche required for full developmental competence.

Subsequent studies have reinforced and expanded upon these findings. Spears et al. [206] showed that mouse primary follicles could be cultured to the Graafian stage and ovulated in vivo to yield offspring, underscoring the intrinsic developmental potential of early-stage follicles when appropriately supported. Similarly, Wang et al. [207] emonstrated that preantral follicles from vitrified mouse ovarian tissue could be matured in vitro to MII and used to produce live-born pups following IVF and embryo transfer. Collectively, these investigations establish murine 3D culture platforms as the most advanced and reliable systems to date for supporting the complete trajectory from isolated follicle to offspring.

In contrast, replicating these outcomes in larger mammals and humans remains challenging. Telfer et al. [208] pioneered a two-step in vitro growth system for human follicles, initially culturing ovarian cortical strips for six days, followed by a four-day culture of isolated secondary follicles in V-shaped microwell plates. In later work, the same group reported that a small number of follicles reached the antral stage and produced oocytes capable of maturing to MII within 20 days, although abnormalities in polar body size were observed [129]. Xu et al. [7] later developed an extended protocol in which ovarian strips were cultured for three weeks prior to the isolation and further culture of secondary follicles for an additional six weeks. This approach yielded oocytes that exhibited a perinucleolar chromatin rim and completed meiosis to MII, with normal spindle formation and polar body morphology. However, concerns remain regarding the potential impact of prolonged culture on genomic imprinting and epigenetic integrity, given the association of in vitro culture conditions with imprinting disorders such as Beckwith-Wiedemann and Prader-Willi syndromes [209]. Thus, although human IVG protocols can yield morphologically mature MII oocytes, no study to date has demonstrated successful fertilization, embryo development, or live birth from entirely in vitro-grown human oocytes.

In non-human primates and large mammals, progress has been made, but remains limited compared to rodents. In vitro culture of secondary [210] and early antral goat follicles [211] has produced MII oocytes capable of fertilization and early embryonic cleavage. However, live births from entirely in vitro folliculogenesis protocols have not yet been achieved in these species. Continued refinement of biomaterials, microenvironmental cues, and multi-step culture protocols will be essential to move closer to the clinical translation of fully in vitro oogenesis.

## 9. Conclusion

In vitro folliculogenesis has progressed substantially over the past decades, driven by a deeper understanding of ovarian physiology and advances in biomaterials, culture systems, and dynamic cultivation technologies. This review has highlighted how the integration of

biochemical, biophysical, and mechanical cues in culture platforms has helped to better replicate the ovarian niche and support the transition from preantral to antral follicles, with increasing success in producing developmentally competent oocytes. Key innovations, including biomimetic scaffolds, perfusion bioreactors, organ-on-a-chip technologies, and targeted cell supplementation, have significantly improved follicle viability, endocrine function, and oocyte quality in vitro. Moreover, these platforms are now being leveraged not only for fertility preservation in cancer patients but also for studying reproductive toxicology, ovarian aging, endocrine disorders, and drug development. Continued refinement and standardization of in vitro systems will be essential to enhance reproducibility, scalability, and clinical translatability.

Nonetheless, despite these promising developments, critical limitations remain that must be addressed to fully realize the clinical and translational potential of these approaches. While recent innovations have undoubtedly advanced the field, several bottlenecks persist. These include the limited capacity to support full maturation of human follicles in vitro, variability in culture outcomes across laboratories, and a lack of standardized protocols that hampers reproducibility and clinical translation. Furthermore, despite their potential, some emerging technologies, such as organoid-based or iPSC-derived folliculogenesis, remain largely exploratory, and current enthusiasm may exceed available experimental validation. As such, a cautious and critical evaluation of each platform's limitations is essential to guide responsible progress in reproductive bioengineering.

Collectively, these achievements mark an important step toward the development of safe, effective, and personalized strategies for fertility preservation and reproductive health research.

## 10. Future perspectives

The field of OTE and in vitro folliculogenesis has witnessed remarkable advances in recent years. Nevertheless, several key challenges must be addressed to enable clinical translation and maximize therapeutic impact. One critical direction for future research is the refinement of dynamic culture platforms, such as perfusion bioreactors and OVoC systems, to more closely replicate the ovarian microenvironment. This includes better integration of endocrine signals, mechanical stimuli, and metabolic exchanges that collectively govern folliculogenesis and oocyte maturation [212,213].

In parallel, innovations in biomaterial design remain essential. The development of responsive, bioactive scaffolds capable of mimicking the ECM and adapting to the mechanical and biochemical needs of growing follicles could substantially enhance culture outcomes [9,214]. These scaffolds should ideally support dynamic remodeling, paracrine signaling, and cellular alignment to sustain follicle integrity over prolonged culture periods.

Additionally, the application of high-resolution single-cell omics, such as transcriptomics, epigenomics, proteomics, and metabolomics, is expected to deepen our understanding of follicular development at an unprecedented level [215–218]. These tools could inform the rational design of stage-specific media formulations, identify novel biomarkers of oocyte quality, and help personalize culture systems based on patient-specific profiles.

Another promising direction is the integration of mathematical and computational modeling to simulate the complex interplay between mechanical forces, signaling pathways, and metabolic gradients within the follicular niche [8]. Such models could guide the optimization of scaffold properties, media composition, and dynamic parameters, while reducing reliance on empirical trial-and-error approaches.

Importantly, cross-disciplinary collaboration between reproductive biologists, bioengineers, data scientists, and clinicians will be crucial for advancing these innovations from bench to bedside. In vitro folliculogenesis has the potential to extend far beyond fertility preservation, offering transformative applications in reproductive toxicology, disease modeling (e.g., ovarian cancer, PCOS), and the study of ovarian aging and endocrine function.

Future platforms should also consider accessibility, scalability, and regulatory compliance, especially for clinical-grade applications. The use of patient-derived cells, including those differentiated from iPSCs, may enable the development of personalized reproductive models and therapies. Furthermore, the incorporation of biosensors and real-time monitoring could improve standardization, safety, and reproducibility.

Beyond technological refinement, the field must also confront broader challenges, including ethical concerns surrounding artificial gametogenesis, regulatory barriers for clinical use, and limited long-term safety data for oocytes or embryos generated entirely ex vivo. Standardization across labs and better-defined outcome measures are urgently needed to ensure reproducibility and enable clinical-grade applications. Collaborative efforts to develop consensus protocols and validation tools will be key to moving beyond proof-of-concept toward robust, translatable systems.

Taken together, these future directions offer a roadmap for transforming in vitro folliculogenesis into a robust clinical and research tool, with wide-ranging applications across reproductive medicine, regenerative biology, and women's health.

## Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Arezoo Dadashzadeh reports financial support was provided by Foundation against Cancer. Saeid Moghassemi reports financial support was provided by Fonds National de la Recherche Scientifique de Belgique. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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